REMARKS

Claims 23-37 are pending in this application. Claims 23-25, 27 and 35-38 are amended. In Claim 23, in the last line of the definition of "R", "lower" is deleted from the expression "lower lower alkoxy". That "lower alkoxy" is the correct expression is evident from the specification at page 28, line 14. Further, "lower alkyl carbonyl and carbonyl lower alkyl, which had inadvertently been included in the definition of R^1 are deleted therefrom. Applicants note with appreciation the helpfulness of the Examiner is bringing this point to their attention. It is respectfully submitted that the foregoing amendments to Claim 23 obviate the rejection thereof under the first paragraph of 35 U.S.C. § 112.

In Claim 24, the expression "Y is >C=Y" is amended to read "X is >C=Y, wherein Y is oxygen" thereby correcting an error in the designation "X" and bringing the claim into agreement with Claim 23. These amendments obviate the rejection of Claim 24 under the second paragraph of 35 U.S.C. § 112. The rejection of Claims 25, 27, 35, 36 and 38 under the second paragraph of 35 U.S.C. § 112 is obviated in each instance by the amendment to relocate " and their pharmaceutically acceptable salts" to the last line of the claim and insert a period thereafter. In Claim 35, "saltz" in the first line is replaced by "said" thereby correcting an obvious inadvertent typographical error. In the first line following the formula "to claim 28" is deleted, thereby correcting an obvious inadvertent misstatement. Finally, Claim 37 is corrected in the definition of "X" in that "a alkoxy" now reads "an alkoxy". It is respectfully submitted that the foregoing amendments do not add new matter to the claims and are fully supported by Applicants' specification. It is further respectfully submitted that the Claims as amended are compliant with the requirements of the first and second paragraphs of 35 U.S.C. § 112. Entry of the amendments and withdrawal of the rejections under 35 U.S.C. § 112 are respectfully requested.

The rejection of Claims 23-28, 30 and 33-36 under 35 U.S.C. § 103(a) as being unpatentable over Harbeson *et al.*, U.S. 2004/0034037, is again respectfully traversed. The indication that Claim 37 is allowable is noted with appreciation. It is assumed that,

with the resolution of the rejections of Claims 29, 31 and 32 under 35 U.S.C. § 112, they will be allowable as well.

In the Final Office Action under reply, the Examiner notes that the comparative data submitted in two reports accompanying the previous Response is not persuasive of the patentability of the Claims under consideration since it is not presented in a Declaration under 37 C.F.R. § 1.132 and did not compare against the compounds considered to be the closest to the Claims under consideration. In response, Applicants are submitting herewith a Declaration under 37 C.F.R. § 1.132 by Dr. Felix Bachmann, one of the co-inventors of the subject application. It is respectfully submitted that the filing of the Declaration after Final rejection is proper in view of the statement in the Final Office Action under reply that the comparative data previously submitted was not persuasive because it was not presented in a Declaration under 37 C.F.R. § 1.132.

In the Declaration, Dr. Bachmann provides a comparative showing of the activity of the claimed compounds against all of the compounds of Harbeson *et al.* suggested for comparison in the Final Office Action. The data provided in the Declaration, in the opinion of Dr. Bachmann, clearly establishes the unexpected activity of the claimed compound in comparison to those disclosed in Harbeson *et al.* and further establishes that one of ordinary skill in the art would not be led to expect that the compounds of the present invention would possess new and advantageous antiproliferative activity in comparison to the Harbeson *et al.* compounds. It is respectfully submitted that the data in the accompanying Declaration establishes that there is no expectation of the new and unexpected activity of the presently claimed compounds and the broad disclosure of Harbeson *et al.*, hence there is no basis for a *prima facie* case of obviousness. Withdrawal of the rejection is respectfully submitted to be in order and such action is respectfully requested.

Accordingly, since the claims under consideration meet the requirements of the first and second paragraphs of 35 U.S.C. § 112 and define patentable subject matter over the citations of record, it is respectfully submitted that his application is in condition for allowance. An early Notice of Allowance allowing Claims 23-37 is courteously solicited. No fees are believed to be necessitated by the instant response. However, should this be in error, authorization is hereby given to charge Deposit Account No. 03-3839 for any underpayment, or to credit any overpayments.

Telephone calls should be address to William H. Epstein, Reg. No. 20,008, at (973) 596-4607 and fax communications should be sent directly to him at (973) 639-6397.

Respectfully submitted,

Gibbons P.C.

By _____
R. Hain Swope - Reg. No. 24,864
Tel. No. (973) 564-4905
Fax No. (973) 639-6357

Date: January 23 , 2008

Please address all correspondence regarding this application to **Customer No. 26345.**
Intellectual Property Docket Administrator
Gibbons P.C.
One Gateway Center,
Newark, New Jersey 07102-5310

PATENT
104610-55275 (22009)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Patent Application of: Eberle et al.			: Art Unit	: 1626
Serial No.	:	10/557,539	: Examiner	: L. Stockton
Filed	:	November 21, 2005		
Title	:	Furazanobenzimidazoles		

DECLARATION UNDER 37 C.F.R. 1.132

1) I, Felix BACHMANN, do hereby declare and say as follows:

That I am a citizen of Switzerland, residing at Grellingerstrasse 38, CH-4052 Basel, Switzerland.

That I received my Master of Science in cell biology from the Biozentrum of the University of Basel in 1989.

That I received a PhD degree at the Friedrich-Miescher Institute (FMI) in Basel in 1996 with a thesis in the cancer area.

That I did post-doctoral studies at the FMI and the Institute for Medical Microbiology of the University of Basel from 1996 to 2000 in the field of programmed cell death (apoptosis) and its function and regulation in cancer.

That I, based on that knowledge, founded together with colleagues in 2000 Aponetics AG, a university spin-off company which focused on the discovery and development of novel anti-cancer compounds, and held the position of the Head of Discovery of said company until the closing down of the company in January 2005.

That I joined Basilea-Pharmaceutica AG in May 2005 and since then have been working there in the field of oncology with a major focus on the characterization and development of compounds derived from the proprietary screening technology of the former Aponetics AG which has been taken over by Basilea-Pharmaceutica AG.

That I am a co-inventor of US Patent Application Serial No. 10/557,539.

That I have read and understood the Office Action issued on April 4, 2007 and the Final Office Action issued December 4, 2007, and the prior art cited therein, especially the reference of HARBESON ET AL (US 2004/003437) and the corresponding PCT publication WO 03/066629.

2) That, under my direction, the tests described below have been made to show the differences between the reference of HARBESON ET AL (US 2004/003437) and the present invention.

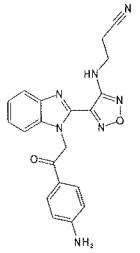
The IC50 values for the inhibition of cell growth/proliferation have been determined versus three different cancer cell lines. The experimental conditions were as follows: Growth/Proliferation (GP) of 3 different cell lines representing clinically relevant tumor types (HeLa: human cervical squamous cell carcinoma, HT1080: human fibrosarcoma, HT-29: human colorectal adenocarcinoma) was determined by the crystal violet staining method according to Gillies, R.J. et al. (Determination of Cell Number in Monolayer Cultures, Analytical Biochemistry, 159: 109-113 (1986)) and Kueng, W. et al, (Quantification of Cells Cultured on 96-Well Plates, Analytical Biochemistry, 182: 16-19 (1989)). Crystal violet retained by the fixed cells was quantified by measuring absorption at 590 nm. IC50 values were calculated by fitting the normalized data to a sigmoidal dose-response model with a variable slope.

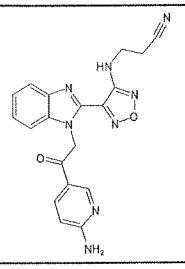
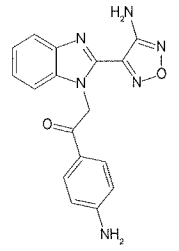
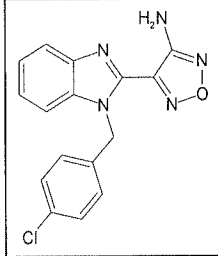
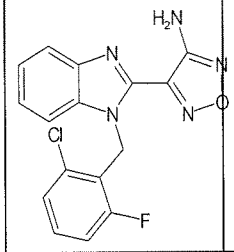
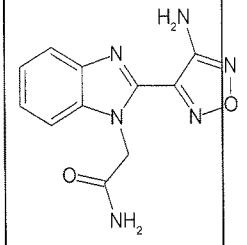
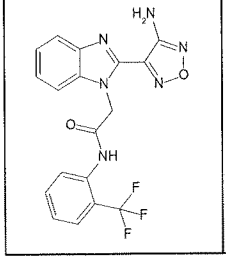
The results are shown in Table 1 and Table 2 below.

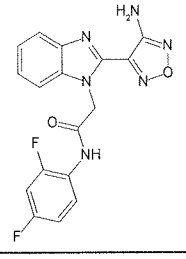
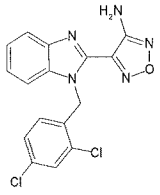
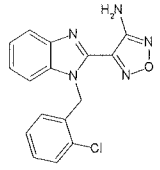
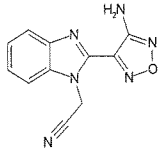
(A) Comparison of compounds of formula (I) of the present application wherein X is >C=Y and Y is oxygen:

It was found that the compounds of Example 50, Example 58 and Example 79 of the present invention, which are representative for compounds of formula (I) according to the present invention wherein X is >C=Y and Y is oxygen, exhibit IC50 values below 50 nM against these cell lines, whereas the IC50 values for the most closely related comparator compounds according to the reference of HARBESON ET AL, i.e. the compounds of the Examples 1, 4, 6, 21 and 24 as well as 12, 13 and 15, were above 1000 nM in all assays.

Table 1. Antiproliferative activity; IC50 values (nM) versus cancer cell lines

Formula	Present Application Serial No. 10/557,539	HARBESON ET AL US 2004/0034037	GP HeLa	GP HT1080	GP HT-29
	Ex. 58	-	18	15	11

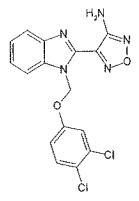
	Ex. 79	-	23	14	17
	Ex. 50	-	44	27	33
	-	***Ex. 1***	> 1000	> 1000	> 1000
	-	***Ex. 4***	> 1000	> 1000	> 1000
	-	***Ex. 6***	> 1000	> 1000	> 1000
	-	***Ex. 21***	> 1000	> 1000	> 1000

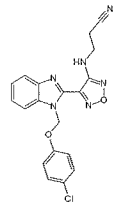
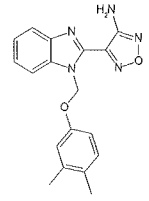
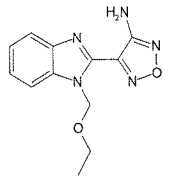
	-	***Ex. 24***	> 1000	> 1000	> 1000
	-	Ex. 15	> 1000	> 1000	> 1000
	-	Ex. 12	> 1000	> 1000	> 1000
	-	Ex. 13	> 1000	> 1000	> 1000

(B) Comparison of compounds of formula (I) of the present application wherein X is -O-:

It was found that the compounds of Example 92, Example 93 and Example 101 of the present invention, which are representative for compounds of formula (I) according to the present invention wherein X is –O-, exhibit IC50 values below 200 nM against these cell lines, whereas the IC50 values for the most closely related comparator compound according to the reference of HARBESON ET AL, i.e. the compound of Example 26, was above 10000 nM in all assays.

Table 2. Antiproliferative activity; IC50 values (nM) versus cancer cell lines

Formula	**Present Application Serial No. 10/557,539**	**HARBESON ET AL US 2004/0034037**	**GP HeLa**	**GP HT1080**	**GP HT-29**
	Ex. 92	-	60	48	43

	Ex. 93	-	150	104	62
	Ex. 101	-	93	77	59
	-	***Ex. 26***	> 10000	> 10000	> 10000

3) From the results of the experiments described above, I conclude that the compounds according to the reference of HARBESON ET AL which are most closely related to the compounds of the present invention do not exhibit antiproliferative activity on cancer cells. This has particularly been shown for the compounds **1, 4, 6** on page 6 and the compounds **21, 24** on page 8 in the HARBESON reference (cf. Table 1) as well as for compound **26** on page 8 in the HARBESON reference (cf Table 2) which the Examiner has suggested for comparison. In my opinion, based on the data in the tables, HARBESON ET AL could not motivate a person of ordinary skills to provide the compounds of the present claims which have significant antiproliferative activity on cancer cells.

4) I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at Basel this 18 th day of December 2007

.....
Dr. Felix Bachmann

ANALYTICAL BIOCHEMISTRY 159, 109–113 (1986)

Determination of Cell Number in Monolayer Cultures

R. J. GILLIES,[1] N. DIDIER, AND M. DENTON[2]

Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523

Received April 15, 1986

Determining the cytostatic or cytotoxic effects of various conditions on monolayer cells requires techniques that are rapid, reproducible, and able to monitor these effects as a function of time. Methods currently used to monitor cytostasis or cytotoxicity are either static or indirect; that is, they are designed to test effects of various treatments either at single time points or on associated cellular processes, such as membrane integrity. Because of these limitations in extant techniques, we undertook this study to improve methods for the rapid determination of cell number in monolayer cultures. We have arrived at conditions of staining cell nuclei with crystal violet under fixed regimens which allow rapid and reproducible quantification of cell number in cultures grown in 24-well miniwells. Quantification is possible by solubilizing the adsorbed dye into a solution of Triton X-100 and determining optical density (O.D.) using spectrophotometry. The present communication documents that O.D. is linearly related to cell number with a sensitivity of ca. 500 cells and that the technique is applicable to study agents which affect cell proliferation.

KEY WORDS: crystal violet; spectrophotometry; growth curves; cytotoxicity.

Most studies of mammalian cell proliferation involve the use of cells with a substrate-attachment requirement, such as fibroblasts or epithelial cells. Such studies often involve investigating a multiplicity of conditions or compounds for their effects on either culture growth or viability. To assay for effects of agents or conditions on cell growth or viability, a technique is required that is rapid, reproducible, and able to process a large number of parallel cultures at various time points.

Culture viability is generally determined using a cytotoxicity assay; that is, one which reproducibly tests identical inocula of cells for their response to putative cytotoxic agents. Cytotoxicity is then generally tested by assaying the integrity of the cellular membrane. These assays include the hydrolysis of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)[3] to a colored product (1,2), the exclusion or inclusion of vital dyes, such as trypan blue (3,4), eosin (5) or neutral red (6), or the release of either radiolabeled substances, such as thymidine (7) or chromium (8), or of enzymes, such as lactate dehydrogenase (9) or aspartate aminotransferase (10). Despite the utility and sensitivity of these different assays, they are incapable of monitoring cytostasis: the proliferation-associated response of cells to growth-perturbing agents. Such studies require the application of a technique to assay for culture growth under a variety of conditions at different time points.

Conventionally, cell number is determined by removing cells from the substratum and counting them with either a hemocytometer

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[2] Present address: Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506.

[3] Abbreviations used; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; crystal violet, *N*-hexamethylpararosaniline; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; HBSS, Hank's Balanced Salt Solution; DMO, 5′,5″-dimethyloxazolidine-2,4-dione.

0003-2697/86 $3.00

or an electronic particle counter. These techniques are laborious and either inaccurate or will not allow observation of cellular morphology. Both of these methods require relatively large numbers of cells. There are a number of techniques which infer cell number from DNA content in crude cell lysates. The most sensitive of these is a fluorescence technique using bisbenzimidazole (11), which can accurately detect as few as 10,000 cells. However, this technique seems to be limited to cell lysates and requires rather sophisticated hardware. It is also ill-suited to the determination of cytotoxicity, since DNA can persist in the culture vessel long after the cytotoxic event.

Recently, a technique has been introduced for the estimation of cytotoxicity using the basic metachromophore, crystal violet (12). Crystal violet (*N*-hexamethylpararosaniline) is a basic dye which stains cell nuclei (13). This technique is extremely sensitive and rapid, yet the fixation and staining conditions, as described, are appropriate only for analysis of cytotoxicity. In the present communication, we introduce an alternative procedure which allows analysis of cell number in growing cultures. This technique is based on estimation of cell number using crystal violet staining of cultures in 24-well culture plates *in situ*.

METHODS

Cell culture. Balb/c-3T3 mouse embryo fibroblasts were obtained from American Type Culture Collection (ATCC CCL 163). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) supplemented with 10% NuSerum (Collaborative Research, Inc). The initial inoculum as received was grown to 70% confluency in 300 cm^2 at which time the cells were frozen in DMEM supplemented with 10% dimethyl sulfoxide (DMSO), and 20% Nu-Serum, at a density of 1×10^6 cells per freezing ampule. The cells were recovered in T75 tissue culture flasks and passed biweekly at an inoculation density of 2×10^5 cells per T75 flask. Experimental cultures were subsequently inoculated into 24-well plates at a density between 8×10^3 and 1×10^5 cells per well, depending on the experiment (see Results). The plating density was consistently achieved by continuous magnetic stirring of the inoculum suspension, using a micropipettor for each 1-ml transfer. In all of the experiments, the first column of wells received no treatment so that it could be used as a standard for comparison between plates. The plates were then incubated at 37°C in humidified, 5% CO_2 atmosphere on a rocker platform to ensure even growth of the monolayer.

Preparation of plates. In initial experiments, it was noticed that cell growth in wells was inhomogenous. The different surface treatments during plate preparation by various manufacturers was one factor that caused uneven cell growth. It was found that plates purchased from Flow laboratories produced the best results. In addition, because of Colorado's dry climate, demagnitization of the plates using a tape head demagnetizer before inoculation was found to be necessary for isotropic cell growth.

Fixation. The cells were fixed with two regimens. In the first, when all wells are fixed at the same time (See experiments 1, 2, 4) they are treated for 15 min with 1% glutaraldehyde (Sigma) in Hanks' Balanced Salt Solution (HBSS). After this time, the cells are stained. In the second protocol, during time course experiments, the cells are fixed for 15 min with 1% glutaraldehyde after which time the samples are kept hydrated with 1 ml of HBSS until staining.

Staining. Samples were stained for 30 min with 1 ml of 0.1% crystal violet solution in deionized water. After this time the plates were submerged in a 1-liter beaker of deionized water and destained for 15 min with a continuous, slow (0.5 l min^{-1}) stream of deionized water introduced at the bottom of the beaker. The plates were then allowed to air-dry. At this time, qualitative results could be observed and photographically documented by noting the amount of stain and growth distribution of cells on a per-well basis.

Absorbance and data analysis. The crystal violet that absorbed onto the cells was solubilized with 0.2% Triton X-100 (Sigma). The absorbance obtained in solution was found to be stable at room temperature for weeks. The colored Triton solution was taken up directly into a Bausch and Lomb Spectronic 2000 uv/vis spectrophotometer using a flow cell at 25°C and the absorbance was measured at 590 nm. Absorbance readings were analyzed by using an in-house Pascal program written for the Apple IIe. This program averages data from each column, normalizes data to control column No. 1, and provides statistical evaluation. In our experience, the standard deviation within a column (four samples identically treated) was consistently 7–10%.

RESULTS

As shown in Fig. 1, the absorbance at 590 nm is proportional to the cell number. In this experiment, the miniwell plates were inoculated with various known amounts of Balb/c-3T3 cells. The entire plate was fixed and stained 7 h after the cells were plated. This time is sufficient to allow the cells to settle and attach to the plate, yet not long enough for cell divisions to occur. Therefore, the degree of staining is indicative of the cell number that was originally plated. In this experiment, the dye was solubilized in 2.0 ml Triton X-100. Plotting these data against absorbance values indicates a linear relationship between O.D. and cell number (Fig. 1). Solubilizing the dye in 0.25–1.0 ml produced similar curves, and the slopes were proportionally larger. To control for differences in plating efficiency, cells were counted *in situ* prior to Triton solubilization of dye. At least 200 cells per well were counted. The cell count was divided by the number of microscopic fields observed to give a value for cells per field, and converted to cells $\times$ cm^{-2} using a stage micrometer. These analyses produced data in good agreement with the predicted cell numbers.

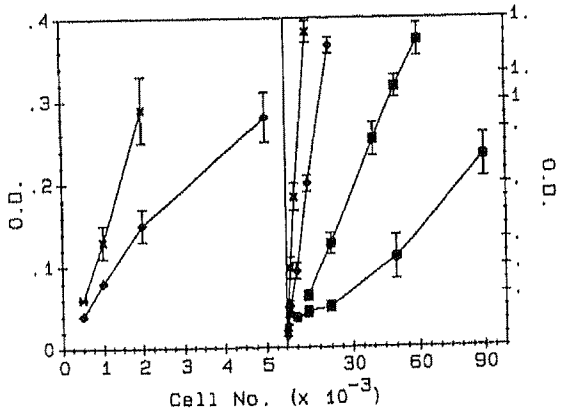

FIG. 1. O.D. as a function of cell number. In these experiments, Balb/c-3T3 cells were inoculated in 24-well miniwells at the concentrations indicated. The entire plate was fixed and stained 7 h after the cells were plated. To illustrate entire range of accuracy, dye was solubilized in 0.25 ml (×), 0.50 ml (♦), 1.0 ml (■), or 2.0 ml (●) prior to reading absorbance at 590 nm. Figure on left is expansion of lower points from figure on right, illustrating linearity in this range.

We then tested this method by growing cells at low density in the presence of various agents for a number of generations prior to determinations of cell number. An example of this is shown in Fig. 2, in which cells were grown in the presence of various concentrations of 5′,5″-dimethyloxazolidine-2,4-dione (DMO). DMO is a weak acid which will cross the plasma membrane in its uncharged form in a pH dependent fashion and acidify cellular compartments (14). Under the conditions of this experiment, DMO is not cytotoxic per se, yet it is cytostatic by virtue of the fact that it inhibits cell growth in proportion to its concentration. As shown in the figure, at an external pH of 6.87, DMO inhibits cell growth at concentrations as low as 20 mM.

Most cytostatic agents will affect the length of the individual cell cycles. To quantitatively monitor this, a techique is needed to determine culture doubling time. As shown in Fig. 3, this technique can also be used in this application. In this experient, the cells were plated at 1 $\times$ 10^4 cells per well in the presence of 10% Nu-Serum. The wells were incubated for various lengths of time and then fixed with 1% glutaraldehyde for 15 min. The wells were kept hydrated in HBSS until the entire plate was fixed, at which time it was stained. These data can be used to determine culture doubling

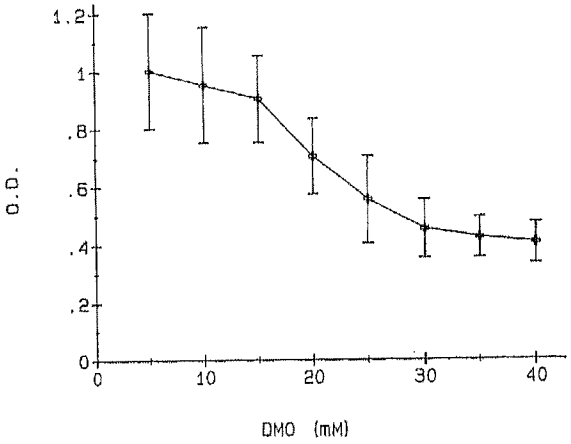

FIG. 2. The effect of DMO on growth in exponential phase BALB/c-3T3 cells. In these experiments, BALB/c-3T3 cells were seeded into 24-well miniwells in complete medium. 24 h later, DMO was added to the concentrations indicated. Cells were grown in this medium at a pH of 6.75 for an additional 48 h, after which time the cultures were fixed, stained, and analyzed as described in the text.

time, which in this case was 18 h. This figure also shows the results of depriving cultures in the presence of 0.2% serum and the effects of restimulating these deprived cultures with 10% serum.

DISCUSSION

The present communication describes a technique for the estimation of cell number in monolayer cultures. It has advantages over conventional techniques in terms of rapidity and sensitivity, although it may not be as accurate or as specific. It is also advantageous in that cellular morphology is preserved and photographs of the cells can qualitatively demonstrate results prior to quantitative analysis.

In our experience, an experiment using the current technique uses about 50 min of total processing time for a 24-well plate. Thirty minutes of this is taken up by the fix/stain/wash step, of which only about 1 min is actually "hands on." Therefore, in terms of "hands on" time, each plate takes about 20 min to inoculate (1 min), treat (2 min), prepare (1 min), and process (15 min). If one uses a microtiter plate reader adjusted to 24-well plates, this could be further reduced. This is to be compared with electronic and hemocytometer counting, which take 75–100 min of "hands on" time for each 24 samples. The present technique therefore represents a four- to fivefold increase in efficiency.

More importantly, however, is the fact that the present technique is extremely sensitive. The data presented indicate that the resolution of this technique is less than 500 cells. The entire range of accuracy (5×10^2—1×10^5 cells) can be achieved by solubilizing the dye in various (0.5–2.0 ml) volumes of the Triton solution (Fig. 1). Particle counting requires a minimum of 1000 cells per 0.5 ml counting volume in a total volume of 5 ml, hence necessitating 10^4 cells. Hemacytometer counting requires a density of 1×10^5 cells/ml in a minimum volume of 100 μl, necessitating also 10^4 cells. The present method therefore represents a 20-fold increase in sensitivity over these

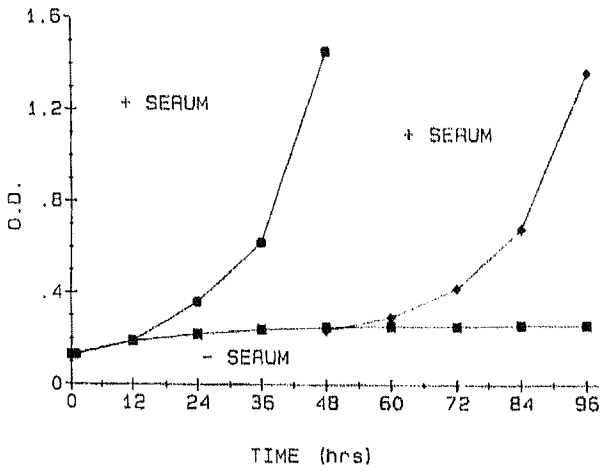

FIG. 3. O.D. (cell number) of cultures as a function of time of serum deprivation or after restimulating with serum. In these experiments, BALB/c-3T3 cells were grown in 24-well miniwells (Flow). At the times indicated, wells were fixed in octaplet with 1% glutaraldehyde, stained with 0.1% crystal violet under fixed regimen, and air-dried. The stain was solubilized in 0.2% Triton X-100 and the O.D. at 590 nm was determined. We have demonstrated in control experiments that the O.D. under these conditions is directly proportional to cell number. In the lower curve, cells growing in 10% serum were deprived at time 0 in medium containing 0.2% serum. In the upper curve, cells that had been deprived for 36 h in 0.2% serum-containing medium were restimulated with medium containing 10% serum.

more conventional techniques for determining cell number.

The major drawback to the present technique is its inability to determine absolute cell numbers because of the difficulties involved in running standard curves. From our experience, plate-to-plate variability in staining of the controls was observed to be as great as 25%. This observation suggests that it would be necessary to run a standard curve on the same plate as the experimental points. This is impractical because of the relatively few numbers of wells on a plate. Comparison of data from plate-to-plate can be achieved by treating the wells in column No. 1 of each plate as controls. Data can then be normalized to column No. 1 on each plate.

It was also noticed that greater accuracy was achieved when experiments were carried out over a number of culture generations, such as those illustrated in Fig. 2 and 3. Notice that, in Fig. 2, the assay was performed over two cell generations and a 2.5-fold difference in O.D. was observed. This could represent one complete doubling of the culture, yet it could signify more than one generation if the background (y intercept) was taken into account. These data should be compared with data from experiment 3; an eightfold difference in O.D. was obtained in three culture generations. The strength of the technique therefore lies in accurately determining relative cell numbers under a wide variety of conditions. Results from these analyses could then be used to presage more quantitative follow-up experiments.

ACKNOWLEDGMENTS

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Quantification of Cells Cultured on 96-Well Plates

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The method for cell number measurement in monolayer cultures by crystal violet staining published recently by Gillies *et al.* (R. G. Gillies, N. Didier, M. Denton (1986) *Anal. Biochem.* 159, 109–113) was modified and significantly improved. The procedure was adapted for use in 96-well plates since the method is inherently very sensitive. Modifications allowed fast and complete solubilization of dye adsorbed by cell nuclei during staining. Since light absorption of the unstained or destained cell layers is negligible, cell number measurements can be performed in the respective wells. Due to these features, multiple assays may be carried out rapidly using standard 96-well plate readers. In addition, it is shown that the sensitivity of the assay can be varied and easily controlled by choosing the appropriate pH during the staining procedure. This increases the flexibility of the method making it useful for determining cell density of a wide range of different cell types.

Direct or indirect measurements of cell numbers are necessary for long-term growth experiments with agents that influence the proliferation of cultured cells. A number of different methods are currently in use, each with its own merits and drawbacks. The culture of cells in 96-well plates is advantageous since fewer cells and lower amounts of expensive growth factors are required. Furthermore, plate readers may then be used for high speed measurements of cell number if the assay is based on spectrophotometric methods. However, an inescapable condition of such a procedure is that it must be sensitive enough to measure only a few hundred cells.

A useful method which fulfills these criteria was described by Mosmann (1). In this case viable cells convert a soluble tetrazolium salt into an insoluble formazan dye. The amount of this product is directly proportional to the cell number. After solubilization with an organic solvent, the optical density (OD)[2] can be measured with a plate reader. The disadvantage of tetrazolium-based assays is that the procedure requires living cells, which can make calibrations and time point comparisons difficult.

Recently, another comparably rapid and sensitive method has been described using fixed cells cultured in 24-well plates; after fixation, cell nuclei are stained with crystal violet solution (2). Subsequently, excess dye is washed out and the crystal violet which has been absorbed by the nuclei is extracted. It was shown that there was a linear relationship between ODs of nuclear extracts and cell numbers (2).

In this study, we present assay modifications based on crystal violet. Our data illustrate that the assay may be easily performed using 96-well plates and used over a broad range of cell numbers.

METHODS

Cell cultures. Human mammary tumor cell lines ZR-75-1, MCF-7, and MDA-MB-231 were obtained from the Mason Research Institute and cultured in IMEM-ZO medium (3) at 37°C in a humified atmosphere with 5% CO_2.

The cells were dispersed from nearly confluent cultures by 0.025% Trypsin (Biocatalyst Ltd.) in 0.02% EDTA solution. Accurate measurements of cell densities were made before seeding on cells suspended in "cellkit 7" solution (TOA Medical Electronics, Kobe, Japan) diluted with two parts of distilled water to ensure absence of cell clumping. Cell counts were performed in a Sysmex CC-108 microcellcounter (TOA Medical Electronics).

For cell growth studies, 50 μl of experimental media (containing twofold concentrations of growth factors) was pipetted into 96-well plates precoated with collagen type IV as described previously (4). Subsequently cell

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[2] Abbreviations used: OD, optical density; IMEM-ZO, improved minimal essential medium, zinc option; DMSO, dimethyl sulfoxide; Mes, 4-morpholineethanesulfonic acid, sodium salt; EGF, epidermal growth factor; β-TGF, transforming growth factor β 1.

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suspension (50 μl), appropriately diluted in serum-free medium without growth factors (see legend of Fig. 2), was added. Thus, homogeneous cell distributions on the bottoms of the well were achieved. The media were changed every fourth day.

Determination of cell numbers by crystal violet staining. Cells were fixed by addition of 10 μl of a 11% glutaraldehyde solution to 100 μl of medium. After being shaken (500 cycles/min for 15 min) on a Sarstedt TPM-2 microshaker (Sarstedt, D-5223 Nümbrecht, FRD), plates were washed three times by submersion in deionized water. Plates were air-dried and stained by addition of 100 μl of a 0.1% solution of crystal violet dissolved in buffers (200 mM) described under Results. After being shaken for 20 min at room temperature, excess dye was removed by extensive washing with deionized water and plates were air-dried prior to bound dye solubilization in 100 μl of 10% acetic acid. The optical density of dye extracts was measured directly in plates using a Dynatech MR 600 microplate reader (Dynatech Laboratories, Inc., Alexandria, VA). The wavelength selected was 590 nm.

MATERIALS

The microwell plates used were obtained from Falcon (No. 3072, Becton–Dickinson, Oxnard, CA). Chemicals were from Sigma (Sigma Chemical Co., St. Louis, MO), epidermal growth factor (EGF) was from Collaborative Research, Inc. (Collaborative Research, Inc., Bedford, MA), and porcine transforming growth factor β 1 (β-TGF) was from R&D Systems (R&D Systems, Minneapolis, MN).

RESULTS

The adaptation of the cell number measurement assay described by Gillies *et al.* (2) required some modifications for use with human tumor cell lines grown in 96-well plates. First, the 0.2% Triton X-100 extraction solution originally proposed did not allow complete destaining of the cells and we therefore tested a number of solvents for this purpose. Among those tried was the acid–isopropanol mixture used by Mosmann (1) to solubilize the formazan product of tetrazolium salt which was more effective than 0.2% Triton but was unfavorable due to its high evaporation rate. Dimethyl sulfoxide (DMSO) proved to be a good solvent for our purposes and resulted in extract of crystal violet having absorbance maximum at 595 nm, which is suitable for the use of plate readers. While the self-absorption of DMSO was found to be reasonably low (data not shown), it had the disadvantage that it took several hours to extract all the dye from stained cells. We obtained the best results with 10% acetic acid, since fixed cells were destained within 10 to 15 min (with shaking) by this solution. Absorbance maximum of crystal violet solutions in 10% acetic acid was found to be 590 nm; in addition, there was little evidence of self-absorption by this solvent.

The crystal violet solution as originally used for staining (2) was prepared with deionized water and yields a pH of 5.0. Since it was possible to solubilize crystal violet with diluted acetic acid (see above), we investigated staining at different pHs to ascertain the effects if any on dye uptakes.

We observed that the intensity of cell staining was strongly dependent on the pH of the crystal violet solution used; at lower pH there was less dye uptake. From two cell lines cells were seeded into 96-well plates at increasing numbers, fixed, and subsequently stained with crystal violet solutions prepared at 200 mM phosphoric acid, pH 2.5, 200 mM formic acid, pH 3.5, and 200 mM Mes, pH 6.0. Under these conditions, the dye uptake of both cell lines (ZR-75-1 and MDA-MB-231) was linearly correlated with cell numbers per well (Fig. 1) and showed correlation coefficients greater than 0.99. However, the relationship between cell number and final OD differed from one cell line to another (Figs. 1A and 1B).

We found that when crystal violet was dissolved in basic solutions cells were more intensively stained. The OD of a cell preparation stained with 0.1% crystal violet solution in 200 mM boric acid, pH 9.0, was two-fold higher than that of a comparable preparation stained at pH 6.0. However, the pH 9.0 solution has to be freshly prepared before use because of instability (data not shown).

Also a significant difference in OD was observed when cells were not dried following fixation and washing. Wet cells took up less dye than air-dried cells (data not shown).

The absorption of unstained or completely destained cell layers was found to be negligible, at least at concentrations up to 5×10^4 cells per well (Fig. 1). Thus our method eliminates the need for corrections to measured values and requires only the inclusion of blanks. Blanks should be derived from cell-free wells on the same plates where the growth experiments were performed. Blank wells were treated in an identical manner to those that contained cells. We also found that coating of culture plates with collagen and/or use of fetal calf serum in the media had no influence on blank values (data not shown).

Results from a representative growth experiment with two estradiol receptor-containing human mammary tumor cell lines are shown in Fig. 2. It demonstrates the accurate performance of the improved assay. The controls grew slowly and reached cell numbers at the end of the experiment which were 4-fold (MCF-7) or 2.5-fold (ZR-75-1) higher than those of the starting levels (upper broken line); β-TGF significantly inhibited the basal growth rate of both cell lines. In contrast, estradiol and

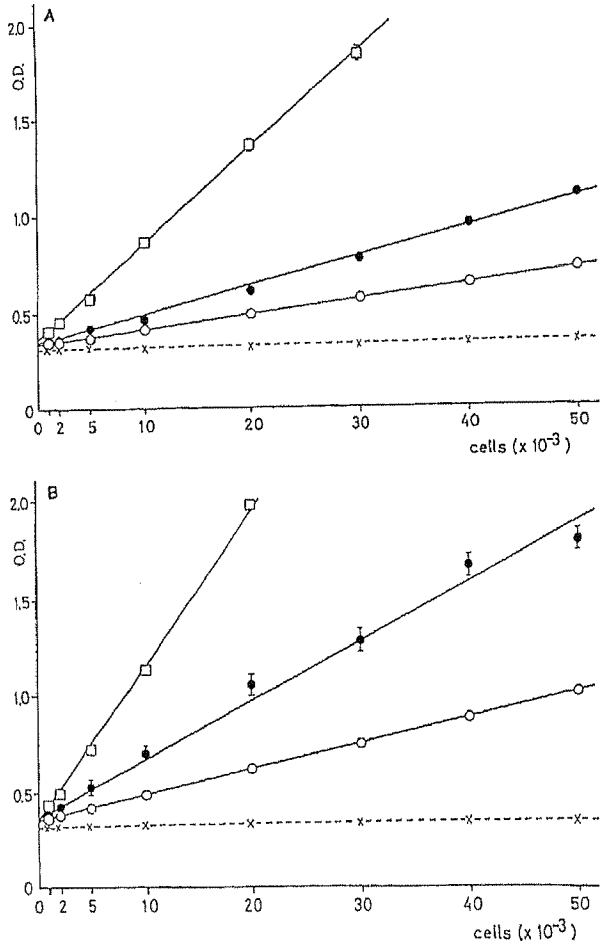

FIG. 1. Correlation of OD with cell number. ZR-75-1 cells (A) and MDA-MB-231 cells (B) were seeded into 96-well plates at the concentrations indicated in media containing 5% fetal calf serum. After 6 h, the cells were fixed and then stained for 20 min with 0.1% crystal violet solutions at pH 6.0 (□), pH 3.5 (●), or pH 2.5 (○). After the excess dye was washed out and being dried the crystal violet adsorbed by the cell nuclei was extracted with 10% acetic acid. The dashed lines (×) indicate the absorption of the destained cells at 590 nm measured with 100 μl of 10% acetic acid per well. Correlation coefficients were greater than 0.99. Bars indicate standard deviations from eight multiplicates.

EGF significantly stimulated the growth of both cell types (Fig. 2).

DISCUSSION

The inherent sensitivity of the assay procedure for determination of cell number using crystal violet staining (2) permits the application of 96-well plates. The use of such plates for experimental protocols has advantages as already outlined when small cell numbers are involved. Furthermore, cell growth studies using expensive growth factor preparations may be undertaken using microwells since the volumes of medium required are minimal.

Concomitant internal standardizations of the procedure are easy as the assay procedure includes a fixation step before cell staining. When cells are seeded out at the beginning of the experiments, an additional plate may be prepared with single, double, and multiple amounts of starting cell numbers. After good attachment, these cells are fixed, washed, dried, and kept so stored until processed at some later stage along with the plates from completed growth experiments. Similarly, if cell numbers have to be determined at different time points during an experiment, the corresponding plates can be processed as described for the standards. Thus, a homogeneous treatment of all cells from an experiment is guaranteed and therefore the results are directly comparable. In contrast to the crystal violet method, the tetrazolium-based assays have the disadvantages that immediate and complete processing and measurement are essential (1).

The data generated by the plate reader need no further correction other than substraction of blank values owing to negligible levels of self-absorption by cell layers at 590 nm (Fig. 1, lower broken line).

We have observed that it is possible to reassay samples that overstained due to highly elevated cell numbers. Under such circumstances the final OD reached or exceeded values of 2.0 and could therefore not be used. To avoid this, we have used a procedure which is outlined below.

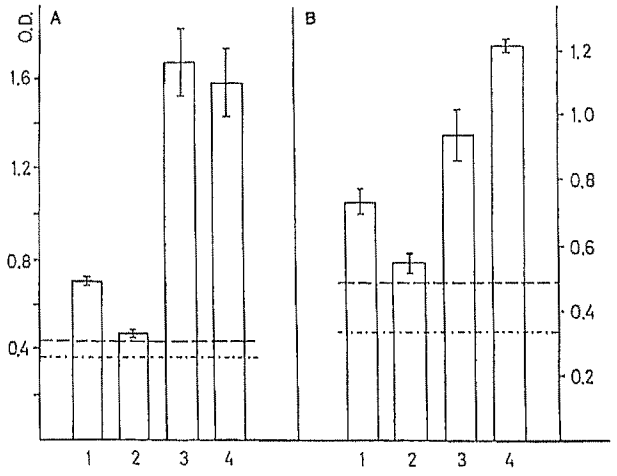

FIG. 2. Effects of β-TGF, estradiol, and EGF on the proliferation of two estradiol receptor-containing human mammary tumor cell lines. Cell lines MCF-7 (A) and ZR-75-1 (B) were seeded in serum-free medium into each well of the 96-well plate coated previously with collagen type IV at 6×10^3 cells/well. All media contained 1 mg/ml fatty acid-free bovine serum albumin, 5 μg/ml iron-free transferrin, 5 ng/ml insulin, 15 mM Hepes, 10 ng/ml Na-selenite, and 5.6 μM (2 mg/liter) phenol red. Control cultures (1) were compared with cultures containing β-TGF (2; 10 ng/ml), estradiol (3; 3×10^{-9} M), or EGF (4; 10 ng/ml). After 7 days, the cells were fixed and stained with crystal violet solution at pH 6.0. The data represent mean values ± standard deviations from quadruplicate determinations. The lower lines indicate the blank values (without cells) and the upper lines the starting cell numbers.

Plates are first completely destained with 10% acetic acid and washed extensively. Then they are either restained while still wet with the original dye solution (see Results) or with dye solutions prepared at a lower pH (Fig. 1). It should be stressed that dilution of dye solutions should not be used since this negates the linear relationship between cell number and OD.

The application of our improved assay procedure is apparent from the results obtained after a growth experiment with two human mammary tumor cell lines (Fig. 2). In defined medium on a collagen substrate, the cells grew slowly without addition of growth factors. In the presence of either estradiol or EGF, the cell proliferation was stimulated in a similar way as described earlier where the experiments were performed in 24-well plates and DNA concentrations were measured (4,5). The inhibitory effect of β-TGF on growth was significant (MCF-7; $P \leq 0.001$; ZR-75-1, $P \leq 0.01$).

In conclusion, this study demonstrates that for growth experiments the crystal violet assay for cell number determination can be performed in the same 96-well plates as the ones used for cell growth. Loss of cells due to manipulations associated with our procedure is likely to be minimal because of the preceding cell fixation. The staining, washing, and dye extraction steps are not critical and the measurement of the OD values is easy and fast if a plate reader is available. The modifications described here increase the flexibility of assay.

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